Synchrotron Infrared Spectromicroscopy as a Novel Bioanalytical Microprobe for Individual Living Cells: Cytotoxicity Considerations

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INTRODUCTION

Recent progress in analytical instrumentation has enabled dramatic advances in gene sequencing and protein identification techniques. Using the information produced by these techniques, the attention of biomedical researchers is now increasingly focused on understanding how chemical species interact in living organisms by the use of imaging techniques that simultaneously provide morphological and chemical information within cells and tissues. Most imaging research has focused on fluorescent labeling to locate a specific chemical event within the cell. However, bond breaking, ionization, and other damage has been shown to occur during excitation with UV, visible, and even the more recent near-IR two-photon techniques.

In contrast, synchrotron-based Fourier transform infrared (SR-FTIR) spectromicroscopy has the ability to monitor the chemistry within an individual living cell without labels and with even lower photon energies. Combining SR-FTIR spectroscopy with microscopy yields a powerful tool for non-destructively probing bio-systems on a small size scale. The sample can be small and/or heterogeneous, for example; individual living cells, microorganisms, and larger biological systems in which local biochemistry may have significant spatial variations.

It is crucial to know if the synchrotron radiation-based mid-infrared (SR-IR) source causes any short- or long-term effects on the living biological samples under study. Mid-infrared photons are significantly lower in energy (0.05 – 0.5 eV) than excitation sources used for fluorescence, implying that photo-induced effects will be minimal. However, to be assured that the SR-IR beam does not perturb living samples via other mechanisms, more detailed studies are required. We recently measured that sample heating from the synchrotron IR beam is minimal (~ 0.5°C).

Here we present the results of in vitro studies to determine if the SR-IR beam causes any detectable immediate or long-term cytotoxic effects on living cells. Four widely accepted assays were used to look for deleterious effects on cells subjected to the SR-IR beam.

MATERIALS AND METHODS

The studies used a human T-1 cell-line from an established aneuploid cell-line derived from human kidney tissue. They were maintained in a standard growth medium at pH 7.4. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were sub-cultured every 3-4 days. T-1 cultures were grown to confluence to ensure para-synchronization. Fluorescence-activated Cell Sorting (FACS) analysis demonstrated that 85% of the cells in these cultures were synchronized to G0/G1 phases. A custom on-stage mini-incubator was used to maintain the proper moisture and growth environment for the cells while allowing *in situ* FTIR spectromicroscopy measurements. Selected cells were exposed at 37°C to the focused synchrotron infrared beam for a specified duration of 5, 10, or 20 minutes. Once completed, fresh growth media was replaced on the dish and it was returned to the standard incubator.

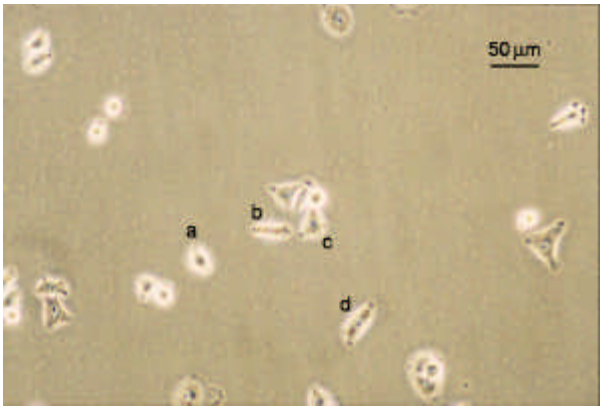

Figure 1. Photograph showing results from alcian blue assays of cells exposed to the SR-IR beam for (a) 5, (b and c) 10, and (d) 20 minutes. Other cells were not exposed and were negative controls. No cells show retention of the blue dye demonstrating that no immediate cytotoxicity is observed.

Negative controls were non-exposed cells located in the same field (internal controls), and therefore experienced the same handling. Positive controls were cells killed by either a 70% alcohol solution or dehydration.

RESULTS AND DISCUSSION

Figures 1-4 show representative photographs for each assay. In no case did we find a result differing with the representative ones shown.

SR-IR beam has no short-term effect on cell viability. Alcian blue assays were carried out as shown in Figure 1. Neither cells exposed to up to 20 minutes of synchrotron IR beam nor nearby non-exposed cells retained the blue dye 6 hours after exposure. This indicates that the SR-IR beam did not produce detectable effects on the viability of exposed cells. Other exposed cells remained free of stain 12 and 24 hours after exposure indicating that their membranes still remained intact. In contrast, dead positive control cells were stained blue as expected, as their membranes had become permeable to the dye molecules.

Cells survive and continue to proliferate days post exposure. The long-term colony-forming assay demonstrates that the exposed cells also continue to proliferate into colonies. The exposed test cells and nearby non-exposed cells proliferated into colonies of similar size (Figure 2), well over fifty cells in ten days. The positive control cells, on the contrary, had detached from the petri dish and disappeared from the field. Since none of the 46 SR-IR exposed test cells developed into colonies with less than 50 cells, we interpret this as an indication that SR-IR beam does not impact cell survival and proliferative activities.

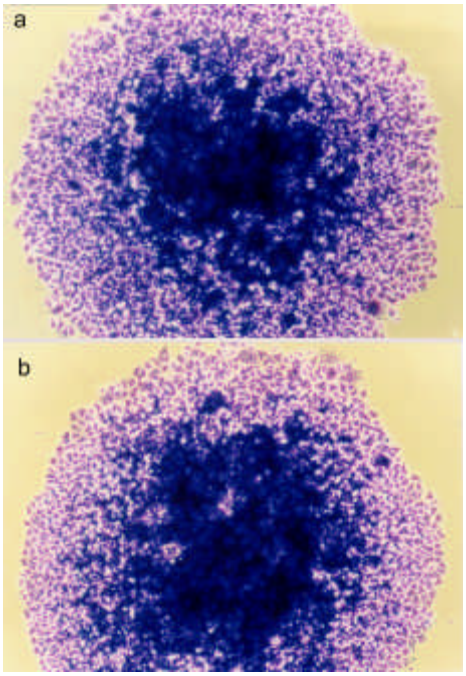

Figure 2. Colony forming from (a) a negative control cell and (b) a test cell exposed to the SR-IR beam for 20 minutes. Both cells proliferated into similar sized colonies after 10 days.

Exposure to SR-IR does not compromise cell-cycle progression. Cell-cycle progression in exposed cells were monitored by the incorporation of BrdU into newly synthesized DNA at 11 hours after cell setup and 10 hours post SR-IR exposure. Both exposed cells and non-exposed controls had reached the DNA synthetic phase (S-phase) of cell- cycle at this 12-hour observation point (Figure 3). The similarities among these immunofluorescent staining of BrdU (and DAPI) labeled cells indicate that the exposed cells are not compromised in their ability to enter their S-phase in the cell cycle after exposure to the SR-IR beam. Furthermore, the lack of BrdU uptake in exposed and control cells at 6 or 24 hours demonstrates that the cell-cycle

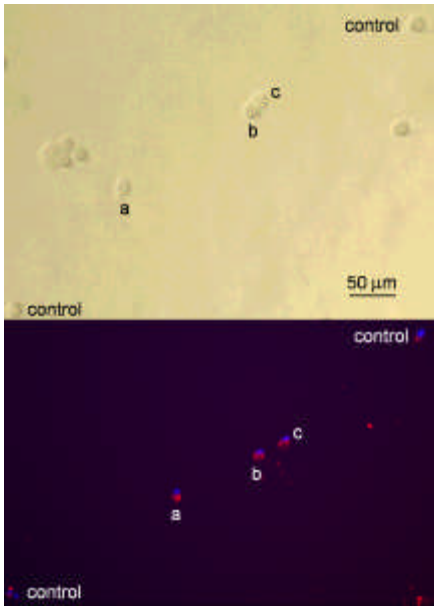

Figure 3. BrdU assay results for cells exposed to the SR-IR beam for (a) 5, (b) 10, and (c) 20 minutes. Two other cells in the field were unexposed and used as negative controls. In the lower panel, blue indicates DNA and red indicates BrdU incorporation during DNA synthesis. All cells show the same incorporation of BrdU into the DNA.

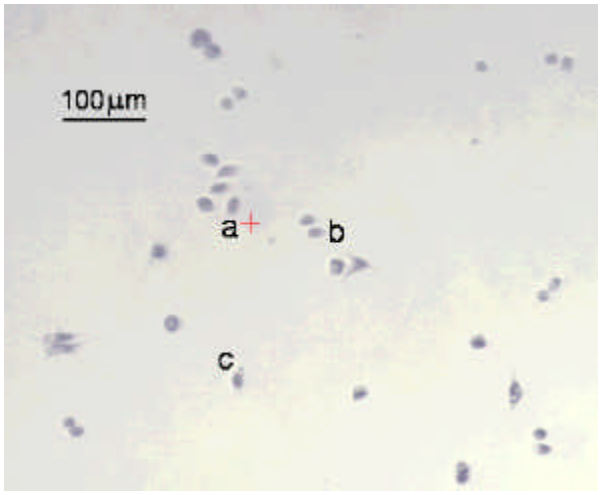

Figure 4. MTT assay results for cells that had been exposed to the SR-IR beam for (a) 5, (b) 10, and (c) 20 minutes. Other cells in the field were unexposed and used as controls. All test and control cells show the same blue color indicating the same level of metabolic activity.

progression of SR-IR exposed cells remains uninterrupted.

ATP and NAD+-associated metabolic activity is not impaired by the SR-IR beam. A two-hour MTT assay was carried out and representative photos of the results are shown in Figure 4. Cells exposed for 20 minutes and nearby non-exposed controls show similar purple-blue stain. On the contrary, tetrazolium salt solution remained yellow in the killed (positive) controls with no purple-blue stain uptake. Results were identical for 5- and 10-minute exposures. This implies that both the exposed and negative control cells produced mitochondrial dehydrogenases during the two-hour MTT assay. Mitochondrial dehydrogenases are associated with the ubiquitous metabolic pathway of glycolysis that generates the critical biomolecules of ATP and NAD+. These results indicate that the SR-IR beam has negligible effects on this important metabolic pathway which provides energy to cells.

In all 4 assays studied we found no detectable changes between cells exposed to the synchrotron infrared beam and nearby non-exposed controls. 267 individual cells were tested with zero showing measurable cytotoxic effects (counting statistics error is 6.1%), with over 1000 control cells used. These results show that the high-brightness mid-IR synchrotron beam is not only non-destructive, but also causes no effects on both the short- and long-term viability, proliferation, and metabolism within living human cells. The results reported here lay an important foundation for future biomedical and biological applications of synchrotron infrared spectromicroscopy, which will complement other biochemistry and microscopy techniques.

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